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Ezrin Regulates E-Cadherin-dependent Adherens Junction Assembly through Rac1 Activation

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Ezrin, a membrane cytoskeleton linker, is involved in cellular functions, including epithelial cell morphogenesis and adhesion. A mutant form of ezrin, ezrin T567D, maintains the protein in an open conformation, which when expressed in Madin-Darby canine kidney cells causes extensive formation of lamellipodia and altered cell-cell contacts at low cell density. Furthermore, these cells do not form tubules when grown in a collagen type I matrix. While measuring the activity of Rho family GTPases, we found that Rac1, but not RhoA or Cdc 42, is activated in ezrin T567D-expressing cells, compared with cells expressing wild-type ezrin. Together with Rac1 activation, we observed an accumulation of E-cadherin in intracellular compartments and a concomitant decrease in the level of E-cadherin present at the plasma membrane. This effect could be reversed with a dominant negative form of Rac1, N17Rac1. We show that after a calcium switch, the delivery of E-cadherin from an internalized pool to the plasma membrane is greatly delayed in ezrin T567D-producing cells. In confluent cells, ezrin T567D production decreases the rate of E-cadherin internalization. Our results identify a new role for ezrin in cell adhesion through the activation of the GTPase Rac1 and the trafficking of E-cadherin to the plasma membrane.

INTRODUCTION

The ezrin, radixin, and moesin (ERM) proteins act as linkers between the plasma membrane and the actin cytoskeleton. They have been implicated in the organization of specific domains of the plasma membrane, such as the apical domain of epithelial cells, the immunological synapse, or the formation of uropods (Berryman *et al.*, 1995; Crepaldi *et al.*, 1997; Serrador *et al.*, 1997; Allenspach *et al.*, 2001; Delon *et al.*, 2001; Roumier *et al.*, 2001). Their functions are regulated by changes in their conformation. ERM proteins exist in the cytoplasm as dormant monomers in which the F-actin cytoskeleton and the plasma membrane binding sites are masked. This closed conformation is due to an intramolecular N- to C-ERM association domain (ERMAD) interaction (Bretscher *et al.*, 1995; Gary and Bretscher, 1995). Thus, abrogation of the N/C-ERMAD interaction is required to "open up" the molecules and to expose their cryptic binding sites (Berryman *et al.*, 1995). Two factors have been implicated in the activation of ERM proteins. The binding to phosphatidylinositol 4,5-biphosphate is required for their interaction with actin in vitro and with membrane proteins in vivo (Nakamura *et al.*, 1999; Barret *et al.*, 2000). In addition, phosphorylation of a conserved threonine residue in the C-ERMAD, T567, inhibits the N/C-ERMAD interaction in vitro (Matsui *et al.*, 1998; Nakamura *et al.*, 1999) and in vivo, converts inactive oligomers to active monomers (Gautreau *et al.*, 2000). Expression of an ezrin mutant, ezrin T567D, which mimics its phosphorylation, induces lamellipodia formation in nonconfluent cells and tufts of microvilli in confluent cells. Moreover, production of ezrin T567D perturbs the organization of epithelial cell monolayers (Yonemura *et al.*, 1999; Gautreau *et al.*, 2000).

A number of studies have implicated ERM proteins in the regulation of cell-cell and cell-matrix adhesion. Suppression of all three ERM proteins with antisense oligonucleotides disrupts cell-cell and cell-matrix adhesion (Takeuchi *et al.*, 1994). Overexpression of ezrin or of its N-terminal domain increased the adhesion of insect cells (Martin *et al.*, 1995). It was further shown that ERM proteins control cell adhesion through different means. For instance, interaction of ezrin with intercellular adhesion molecule (ICAM)-2 is important for the activation of natural killer cells (Helander *et al.*, 1996), the positioning of ICAM-3 in the uropod of T lymphocytes depends on moesin (Serrador *et al.*, 1997), and exclusion of CD43 from the cell-cell contact area during formation of the immunological synapse requires its interaction with ERM

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proteins (Allenspach *et al.*, 2001; Delon *et al.*, 2001; Roumier *et al.*, 2001). ERM proteins have also been shown to control adhesion through the Rho GTPase pathway. ERM proteins are involved in assembly of stress fibers and in the formation of focal adhesions upon activation of the small GTPases Rho and Rac in permeabilized fibroblasts (Mackay *et al.*, 1997). The binding of the TSC1 tumor suppressor hamartin to activated ezrin has been implicated in the activation of RhoA and formation of focal contacts (Lamb *et al.*, 2000). Phosphorylation of ezrin by the Rho kinase ROCK is required for Rho-induced focal adhesion assembly (Tran Quang *et al.*, 2000).

Herein, we report that ezrin T567D expression induces morphological changes in MDCK epithelial cells, leading to lamellipodia formation and perturbed cell-cell contacts. We found that expression of ezrin T567D leads to the activation of the GTPase Rac1, but neither that of RhoA, nor of Cdc 42. This activation of Rac1 perturbed the localization of E-cadherin to the plasma membrane. E-cadherin accumulated in intracellular compartments and its delivery to the plasma membrane was delayed during junction assembly. Our observations provide the first cue on the role of ezrin in E-cadherin–dependent cell-cell junction assembly.

MATERIALS AND METHODS

Cell Culture

The T23 clone of the parental dog kidney-derived epithelial Madin-Darby canine kidney (MDCK) cell line (BD Biosciences Clontech, Palo Alto, CA), which expresses the tetracycline-repressible *trans*-activator (Barth *et al.*, 1997), was used. Cells were grown in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, at 37°C in 10% CO_2. Geneticin (0.4 mg/ml) (Sigma-Aldrich, St. Louis, MO), indicated amounts of doxycyclin (Sigma-Aldrich) and hygromycin B (0.2 mg/ml) (Calbiochem, La Jolla, CA) were added to the growth medium of the transfected MDCK cells. To obtain polarized monolayers, 3×10^6 MDCK cells were plated on 24-mm Transwell filters (Corning Glassworks, Corning, NY) and grown for 4 d.

Plasmid Constructs and Transfection

VSVG (*v*esicular *s*tomatitis *v*irus *g*lycoprotein)-tagged-ezrin T567D and VSVG-tagged-ezrin wild-type (Gautreau *et al.*, 2000) were subcloned into the *Not*I site of the tetracycline-repressible pTRE vector (Tet-Off; BD Biosciences Clontech). Stable and transient transfections were performed by electroporating cells by using an electrical pulse of 0.240 kV and 950 μF (Bio-Rad, Hercules, CA). The plasmid conferring the resistance to hygromycin was from BD Biosciences Clontech. The myc epitope-tagged dominant negative N17Rac1, and dominant active V12Rac1 plasmids were obtained from Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA). The rhotekin Rho-binding domain (amino acids 7–89) fused with glutathione *S*-transferase (GST) was from Dr. Schwartz (Ren *et al.*, 1999). The PAK CRIB-encompassing domain (amino acids 70–118) fused with GST was from Dr. Lowe (Thompson *et al.*, 1998).

Antibodies

Mouse monoclonal anti-human E-cadherin, anti-human Rac1, and anti-human Cdc42 antibodies were obtained from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-human RhoA antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rat monoclonal anti-human E-cadherin (clone DECMA-1) and rabbit polyclonal anti-human β-catenin were obtained from Sigma-Aldrich. Rabbit polyclonal anti-α-catenin was from Chemicon International (Temecula, CA). Mouse monoclonal anti-tubulin was from Amersham Biosciences UK (Little Chalfont, Buckinghamshire, United Kingdom). Mouse monoclonal anti-transferrin receptor was from Zymed Laboratories (South San Francisco, CA). Mouse monoclonal anti-Na^+/K^+ ATPase was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-ezrin antibodies were described previously (Algrain *et al.*, 1993). Myc-tagged proteins were detected with a mouse monoclonal anti-human myc antibody (clone 9E10). The mouse monoclonal anti-VSVG antibody (clone P5D4) was described previously (Kreis, 1986).

Immunofluorescence

Cells were cultured on glass slides, washed with cold phosphate-buffered saline (PBS), fixed, and permeabilized with methanol/acetone (1:1, vol/vol) for 5 min at −20°C, and then incubated with 15% fetal bovine serum in PBS for 1 h at room temperature. Alternatively, cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X 100. Primary and secondary antibodies were incubated for 1 h each. The nuclei were labeled using 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Cells were mounted in PBS/glycerol (1:1 vol/vol) and viewed by epifluorescence (Leica DMRA). Imaging was performed using MetaView.

GTPase Activity Assays

The effectors rhotekin and PAK were used to affinity-precipitate endogenous cellular GTP-Rho, and GTP-Rac and Cdc 42, respectively (Ren *et al.*, 1999). For affinity-precipitation of GTP-bound GTPases, cells were first washed with ice-cold PBS and incubated with the lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% Na DOC, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, containing a cocktail of protease inhibitors). The cleared lysates were incubated with GST-effector binding domain (20 μg) on beads for 1 h on ice. The beads were washed four times with 50 mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, containing a cocktail of protease inhibitors. Beads were resuspended in reduced SDS sample buffer and heated at 95°C for 10 min. Samples were run on 13% SDS-PAGE gels and transferred to membranes. Immunodetection was performed with anti-Rho, -Rac, or -Cdc 42 antibodies. The amount of GTP-bound GTPases was normalized to the total amount of GTPases present in whole cell lysates. Scanning and densitometric analyses were performed with the ImageQuant image analysis system (Amersham Biosciences UK).

Immunoprecipitation and Immunoblotting

Cell lysates extracted in cold radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% deoxycholate, 0.2% SDS, 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, containing a cocktail of proteases inhibitors) were resolved by 10% or 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose (Millipore, Bedford, MA), immunoprobed, and detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Scanning and densitometric analyses were performed with the ImageQuant image analysis system. For immunoprecipitations, cleared lysates were incubated with either 2 μg of the antibody of interest or an irrelevant antibody for 2 h at 4°C. After incubation, protein G beads (Sigma-Aldrich) were washed four times with 1 ml of lysis buffer, suspended in Laemmli buffer and the proteins resolved by 10% or 7.5% SDS-PAGE.

Metabolic Labeling and Pulse Chase

Metabolic labeling was performed in DMEM without Met and Cys complemented with 250 Ci/ml ^{35}S-labeled Met and Cys (Redivue Promix, Amersham Biosciences, Amersham, United Kingdom). Cells were radiolabeled for 20 min and chased for the indicated time in standard DMEM containing 10% fetal bovine serum. After immunoprecipitation, lysates were resuspended in reducing sample buffer, boiled for 5 min, and resolved by 7.5% SDS-PAGE. The ^{35}S signal was enhanced by incubating gels in 1 M salicylate for 20 min.

Dried gels were exposed to a phosphoscreen for 3 d. Signals were quantified using a STORM 860 PhosphorImager and ImageQuant software.

Cell Surface Biotinylation and Transport Assay

Cell surface proteins were biotinylated by incubating the cells with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce Chemical) for 1 h at 4°C and free biotin was quenched with a blocking solution (50 mM NH_4Cl in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$). Cells were then either directly extracted in a RIPA buffer, or stripped to remove the extracellular bound biotin with 50 mM glutathione, 75 mM NaCl, 75 mM NaOH and 2% bovine serum albumin, at 4°C, and RIPA extracted. To measure the intracellular pool of biotinylated proteins, biotinylated cells were incubated at 37°C for 1 h to allow internalization of biotinylated proteins, and then stripped and extracted (Le *et al.*, 1999). Cell extracts were centrifuged and incubated with streptavidin magnetic beads (Dynal, Olson, Norway) to collect biotinylated proteins. These samples were then analyzed by SDS-PAGE and immunoblotting was performed using antibodies against E-cadherin, Na^+/K^+ ATPase or transferrin receptor.

Three-dimensional Cultures

The tubulogenesis assay used was described previously (Crepaldi *et al.*, 1997). Collagen type I gels were prepared as follows: 1 part DMEM 10×, 1 part $NaHCO_3$ (37 g/l), 1 part fetal bovine serum were mixed with 3.5 parts of a suspension of 3×10^5 cells/ml and 3.5 parts of type I collagen at 5 mg/ml (BD Biosciences, Franklin Lakes, NJ) at room temperature. The gels were covered with culture medium supplemented with 100 U/ml hepatocyte growth factor (HGF), and the cells grown for 6 d. Photographs were taken with an epifluorescence microscope (Leica) after paraformaldehyde fixation.

Cell Proliferation

Cell proliferation was measured with a cell proliferation kit (CellTiter 96; Promega, Madison, WI). Cells (5×10^3) were plated as triplicates in 96-well plates and cultured for the indicated times. Then, cells were incubated for 4 h in the presence of the substrate, lysed, and absorbance was recorded at 570 nm (SpectraMax; Molecular Devices, Sunnyvale, CA).

RESULTS

Ezrin T567D Induces Morphological Changes When Expressed in MDCK Cells

We have previously shown that phosphorylation of threonine 567 regulates the transition from inactive ezrin oligomers to active monomers and that expression of a pseudophosphorylated variant of ezrin, ezrin T567D, induced dramatic morphological changes and impaired cell-cell adhesion in LLC-PK1 cells (Gautreau *et al.*, 2000). To investigate the effects of ezrin T567D on cell adhesion we cotransfected MDCK cells expressing the tetracycline transactivator with either the plasmids encoding ezrin T567D, or wild-type ezrin and the plasmid conferring the resistance to hygromycin. The exogenous proteins were tagged at the COOH terminus with a VSVG epitope. For each construct, several independent clones were isolated. Three of them were characterized and used in the subsequent experiments. Results shown are for one representative clone of MDCK cells expressing either ezrin T567D or wild-type ezrin. Immunoblotting with an anti-VSVG antibody showed that these clones expressed ezrin upon omission of doxycyclin from the culture medium, whereas

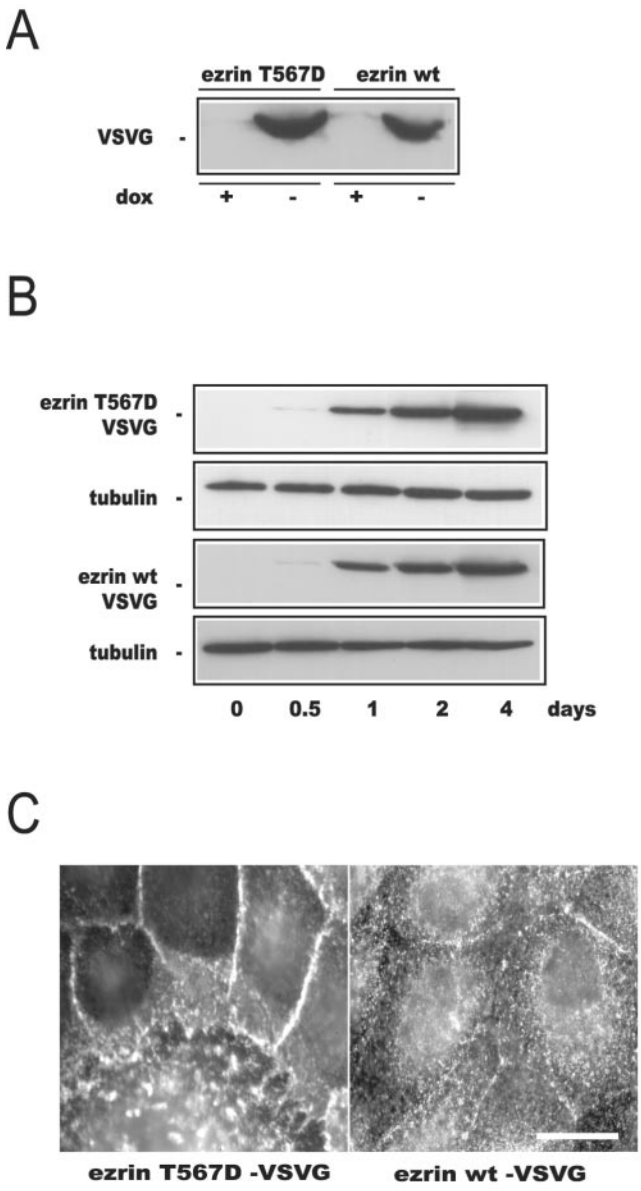

Figure 1. Inducible expression of ezrin T567D in MDCK cells. (A) MDCK cells transfected with cDNAs encoding ezrin T567D or wild-type ezrin were grown in the presence (+ dox) or absence (− dox) of doxycyclin for 4 d. Total cell lysates were analyzed by immunoblotting with an anti-VSVG antibody. (B) MDCK cells expressing ezrin T567D or wild-type ezrin were grown for the indicated times in the absence of doxycyclin. Total cell lysates were analyzed by immunoblotting with an anti-VSVG antibody. Equal loading of the samples was checked by immunoblotting with a tubulin antibody. (C) Immunofluorescence staining of MDCK cells grown in the absence of doxycyclin for 4 d, by using an anti-VSVG antibody. Bar, 10 μm.

no expression was detected when cells were grown in presence of doxycyclin (Figure 1A). Expression of ezrin T567D and wild-type ezrin was detected after 12 h of induction, and increased up to 4 d (Figure 1B). Immuno-

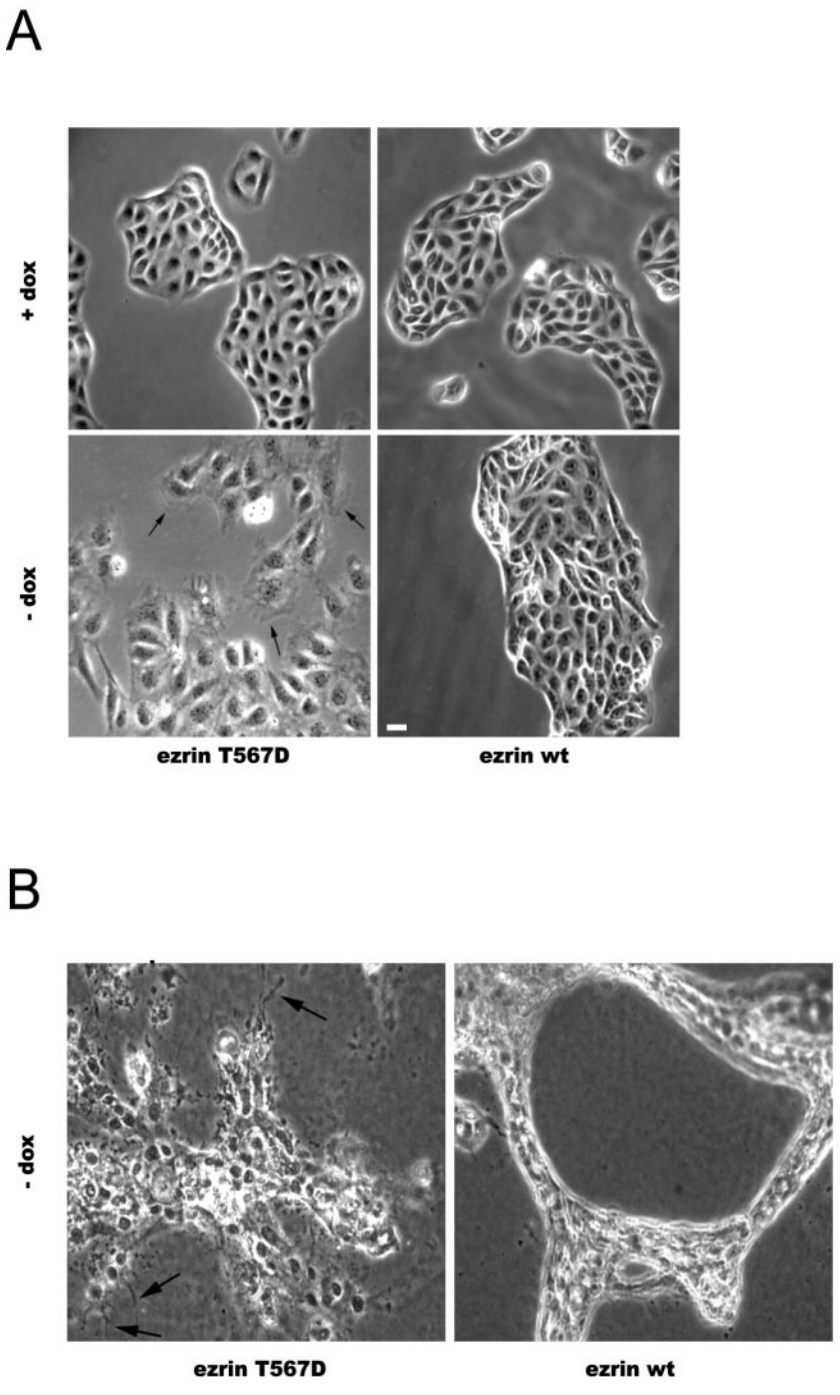

Figure 2. Morphogenic effects of ezrin T567D in MDCK cells. (A) Phase contrast images of MDCK cells grown at low density in the presence (+ dox) or absence (− dox) of doxycyclin for 4 d. Bar, 10 μm. (B) Tubulogenesis assay performed in a collagen type I gel in the presence of HGF and in the absence of doxycyclin for 6 d. The cells producing wild-type ezrin form organized tubules, whereas MDCK cells producing ezrin T567D fail to form tubules. Numerous membrane protrusions are observed in cells producing ezrin T567D (arrows). Bar, 25 μm.

fluorescence stainings indicated that wild-type ezrin was concentrated in the small microvilli of the dorsal cell surface. However, ezrin T567D was also concentrated at cell-cell contacts. Ezrin T567D induced the formation of irregular microvilli at the cell surface (Figure 1C).

Examination of cell morphology by phase contrast microscopy showed that cells overexpressing wild-type ezrin formed regular epithelial islets (Figure 2A). In contrast, striking morphological effects were observed in cells producing ezrin T567D. In the islets, cells were flat, loosely adherent to each other and they displayed numerous lamellipodia. To determine whether production of ezrin T567D affected the formation of multicellular structures, cells were assayed for tubulogenesis in a three-dimensional collagen type I gel. Trypsinized cells were embedded in a collagen type I gel and cultured for 6 d in

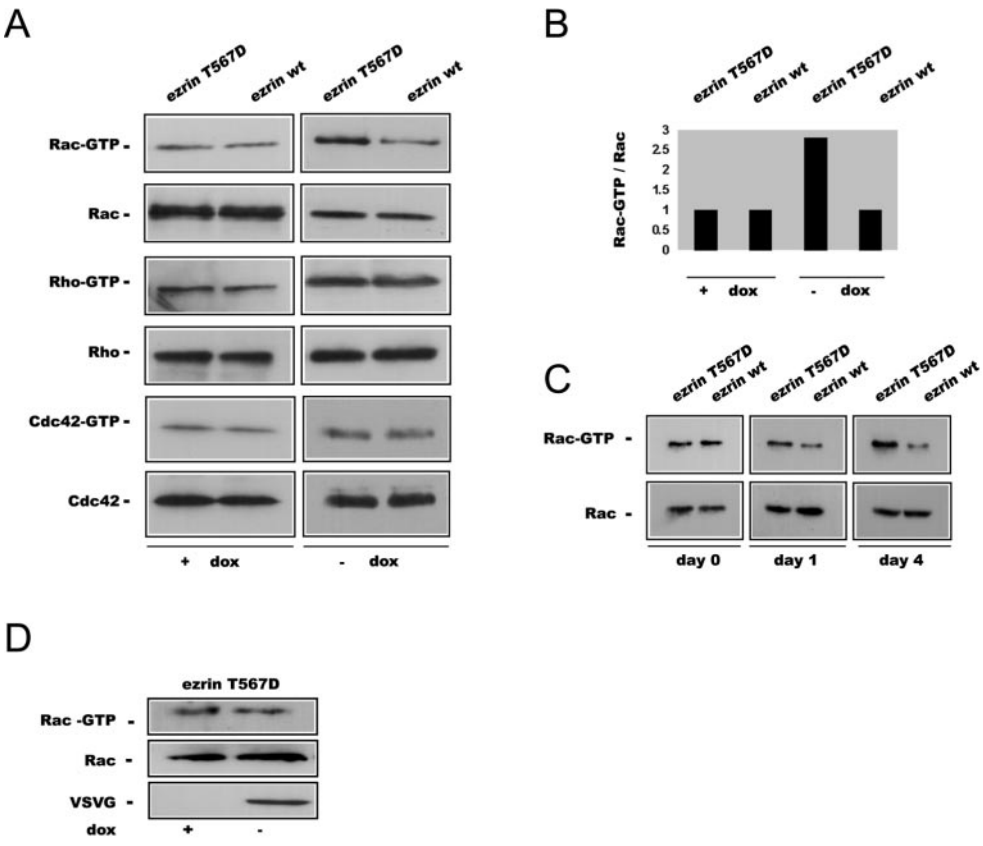

Figure 3. Ezrin T567D activates the GTPase Rac1. Lysates from cells grown in the presence (+ dox) or absence (− dox) of doxycyclin for 4 d were incubated with a GST-PAK fusion protein for the Rac1 and Cdc 42 activity assays, or with a GST-rothekin fusion protein for the RhoA activity assay. (A) The amount of active GTP-bound Rac1, Cdc 42, or RhoA was analyzed by immunoblotting with anti-Rac1, -Cdc 42, or -RhoA antibodies after the affinity precipitation step. Total amounts of Rac1, Cdc 42, or RhoA were determined by immunoblotting of the cell lysates. (B) The densitometric quantification of Rac1 activity shown in A is representative of four independent experiments. Relative Rac1 activity was calculated from the amount of GTP-bound Rac1 normalized to the amount of total Rac1 in cell lysates. An arbitrary unit of 1 was given for Rac1 activity in control cells. Rac activity is induced 2.5-fold in cells producing ezrin T567D compared with cells producing wt ezrin. (C) Immunoblotting with anti-Rac1 antibodies of GTP-bound Rac1 and total amount of Rac1 in lysates prepared from cells grown in absence of doxycyclin for the indicated time. (D) MDCK cells were grown for 4 d after confluence with (+ dox) or without (− dox) doxycyclin. Immunoblotting with an anti-Rac1 antibody shows a similar amount of GTP-bound Rac1 in both culture conditions.

the presence of HGF and in the absence of doxycyclin. Unlike cells producing wild-type ezrin, ezrin T567D-producing cells did not form organized tubules but formed cellular aggregates with randomly distributed membrane protrusions (Figure 2B). Thus, production of ezrin T567D in MDCK cells altered their ability to establish functional cell-cell contacts.

Expression of Ezrin T567D in MDCK Cells Activates the Small GTPase Rac1

Because small GTPases of the Rho family are regulators of lamellipodia membrane protrusions (Hall, 1998), we asked whether the membrane extensions observed in MDCK cells producing ezrin T567D were due to an activation of these GTPases. GST-PAK and GST-rhotekin fusion proteins were used to measure the GTP loading of endogenous Rac1-, Cdc42-, and RhoA-GTPases, from cell lysates of cells expressing either ezrin T567D or wild-type ezrin and grown with or without doxycyclin for 4 d (Figure 3A). Based on four independent experiments, we found that the level of Rac1-GTP was induced ~2.5 fold in cells producing ezrin T567D, compared with cells producing wild-type ezrin (Figure 3, A and B). In contrast, no change in the level of the RhoA- or Cdc42-GTP was detected in the cells expressing ezrin T567D compared with cells expressing wild-type ezrin (Figure 3A). This change in Rac1 activity was readily observed after 1-d omission of doxycyclin and increased up to 4 d (Figure 3C), as did the expression level of ezrin T567D (Figure 1B). This indicated that Rac1 activity correlated with the expression of ezrin T567D. To rule out the possibility that the Rac1 activation observed in MDCK cells producing ezrin T567D was due to a difference of growth rate with cells expressing wild-type ezrin, we measured the cell growth of the different clones up to day 4. No significant differences in the growth rate were observed between cells producing ezrin T567D and wild-type ezrin (data not shown).

Because lamellipodia were mainly observed in nonconfluent MDCK cells producing ezrin T567D, we asked whether activation of the GTPase Rac1 was sustained in confluent cells producing ezrin T567D. MDCK cells were grown at confluence on filters in the absence of doxycyclin in the medium for 4 d. In these culture conditions, the level of Rac1-GTP was similar to that of control cells (Figure 3D). Together, these data indicated that production of constitutively activated ezrin leads to the activation of the GTPase Rac1 in subconfluent cells.

Production of ezrin T567D in MDCK Cells Perturbs E-Cadherin Localization in a Rac1-dependent Manner

Because ezrin T567D production led to loosening of cell-cell contacts and impaired tubulogenesis (Figure 2), we examined whether it affected the E-cadherin–dependent cell-cell adhesion. Two pools of E-cadherin were observed in the ezrin T567D repressed cells and in cells producing wild-type ezrin. E-cadherin was detected at the cell-cell contacts and in intracellular compartments (Figure 4A). In contrast, in cells producing ezrin T567D we observed a strong accumulation of E-cadherin in intracellular compartments and a weak staining at the plasma membrane.

To determine whether this decrease in E-cadherin at the plasma membrane of MDCK cells producing ezrin T567D was due to the activation of the GTPase Rac1, we overexpressed in these cells the dominant negative form of Rac1, Rac1N17. On average, 150 RacN17-positive cells were examined in three independent experiments. We observed that expression of Rac1N17 restored the localization of E-cadherin at the cell-cell contacts in 92% of the Rac1N17-positive cells (Figure 4B). Conversely, Rac1V12, a constitutively active mutant of Rac1, induced the rounding of cells (Figure 4B). Furthermore, 87% of Rac1V12 positive cells showed a strong intracellular accumulation of E-cadherin. Thus, activation of the GTPase Rac1 in cells producing ezrin T567D impaired the localization of E-cadherin to the plasma membrane.

E-Cadherin Accumulates in Intracellular Compartments of MDCK Cells Producing Ezrin T567D

To confirm the immunofluorescence data indicating a decrease in E-cadherin at the plasma membrane, we measured biochemically the level of E-cadherin present at the surface of the cells producing ezrin T567D, or wild-type ezrin. Cells were incubated at 4°C with biotin, and the amount of biotinylated E-cadherin was determined after immunoprecipitation with anti-E-cadherin antibodies and Western blot analysis with streptavidin coupled to peroxidase. The level of E-cadherin at the cell surface was 2.0-fold lower in cells producing ezrin T567D, compared with control cells (Figure 5A). To determine whether this reduced level corresponded to a decrease in the total amount of E-cadherin, we evaluated the amount of E-cadherin in the different clones tested. As shown in Figure 5B, comparable levels of E-cadherin were present in the cells expressing either ezrin T567D or wild-type ezrin. To confirm that the reduced amount of E-cadherin at the plasma membrane was due to an accumulation of E-cadherin in intracellular compartments and not to an increased instability, we measured the half-life of E-cadherin. Pulse-chase analysis performed with MDCK cells producing ezrin T567D grown in presence or absence of doxycyclin in the medium indicated that E-cadherin half-life was similar in both culture conditions, 4.2 and 3.8 h, respectively (data not shown). Together, these results indicated that production of ezrin T567D in MDCK cells impaired the delivery of E-cadherin to the plasma membrane.

E-Cadherin Delivery to the Plasma Membrane Is Delayed in Cells Producing Ezrin T567D

Because adherens junctions are dynamic structures continuously assembling and disassembling, MDCK cells induced to express ezrin T567D, or wild-type ezrin were challenged by a calcium switch (Figure 6, A and B). Depletion of extracellular calcium disrupted epithelial cell-cell contacts and induced E-cadherin internalization in both cell lines (Figure 6A, time 0). On restoration of physiological level of extracellular calcium, adherens junctions progressively reestablished, with a concomitant increase in E-cadherin localization at the plasma membrane. After 2 h in high calcium medium, E-cadherin was significantly recruited at the cell surface of cells producing wild-type ezrin, whereas this delivery was significantly inhibited in ezrin T567D-producing cells (Figure 6, A and B). After 5 h in the high calcium medium, E-cadherin at the cell contacts further increased in cells expressing wild-type ezrin, whereas it remained lower at the cell surface of the cells producing ezrin T567D (Figure 6, A and B). A neosynthetized pool of E-cadherin was not necessary for reestablishment of cell-cell contacts because this occurred even in the presence of cycloheximide (data not shown). These data confirmed that the expression of ezrin T567D delayed E-cadherin-based junction assembly and E-cadherin delivery to the plasma membrane.

Ezrin T567D Slows Down E-Cadherin Internalization in a Polarized Monolayer

MDCK cells producing ezrin T567D were not able to form a functional epithelium when assayed for tubulogenesis, indicating that the cells were not able to establish functional contacts. We thus examined whether production of ezrin T567D had any effects on E-cadherin present at the cell-cell contacts of epithelial cells grown on filters. In these conditions, ezrin T567D-producing cells formed a confluent monolayer with E-cadherin localized at the plasma membrane (Figure 7A). As assessed by biotinylation of cell surface proteins, a similar amount of E-cadherin was present at the cell surface of cells producing ezrin T567D or wild-type ezrin (Figure 7A). By selective biotinylation of the apical or basolateral side of the cells grown on filters, we observed that E-cadherin localized correctly at the basolateral pole (Figure 7B). Na^+/K^+ ATPase was used as a control of basolateral membrane marker excluded from the apical membrane domain. Thus, ezrin T567D production did not alter the membrane-targeting events required for a polarized E-cadherin distribution. We therefore determined the ability of E-cadherin to be internalized in cells grown on filters by using a quantitative cell surface biotinylation assay (Figure 7C). The amount of internalized E-cadherin was reduced in cells expressing ezrin T567D compared with control cells. This down-regulation of internalization was also observed with the transferrin receptor. As a control, no internalized pool of the basolateral membrane protein Na^+/K^+ ATPase was detected.

DISCUSSION

We found herein that production of an ezrin mutant mimicking its constitutive phosphorylation, leads to formation of cell lamellipodia and disruption of cell-cell contacts in kid-

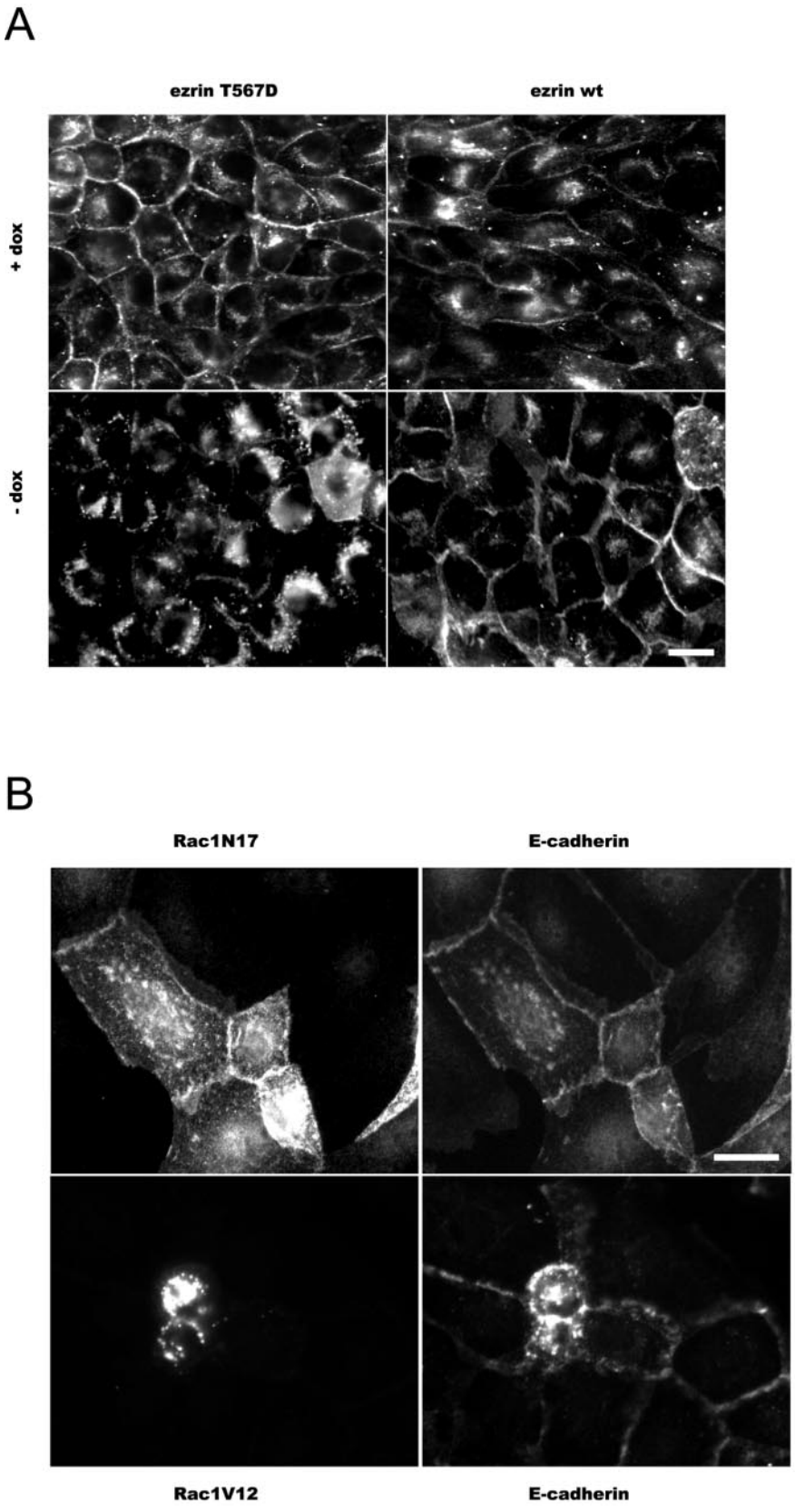

Figure 4. Activation of the GTPase Rac1 in MDCK cells producing ezrin T567D perturbs E-cadherin localization. (A) Immunofluorescence analysis with anti-E-cadherin antibodies of MDCK cells grown in the presence (+ dox) or absence (− dox) of doxycyclin for 4 d. An intracellular pool of E-cadherin accumulates in cells producing ezrin T567D with a concomitant decrease of E-cadherin at the plasma membrane. (B) MDCK cells producing ezrin T567D were transiently transfected with cDNAs coding for Rac1N17 (top) or Rac1V12 (bottom). Double immunofluorescence staining of the cells was performed with anti-E-cadherin antibodies (right) and anti-myc antibodies (left). Bar, 10 μm.

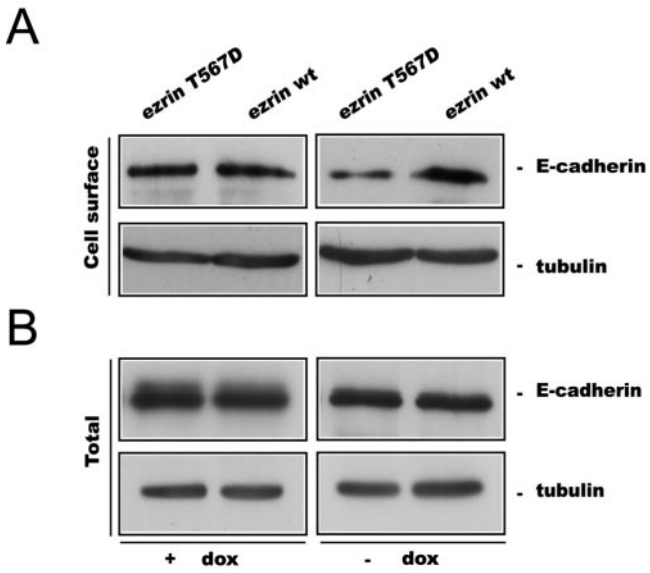

Figure 5. Ezrin T567D decreases the amount of E-cadherin at the plasma membrane. (A) MDCK cells were biotinylated at 0°C for labeling of cell surface proteins. Biotinylated proteins were recovered on streptavidin beads and analyzed by immunoblotting with anti-E-cadherin antibodies. Immunoblotting with an anti-tubulin antibody served as loading control. (B) Total amounts of E-cadherin and tubulin were determined by immunoblotting. The total amount of E-cadherin was similar in MDCK cells producing ezrin T567D or wild-type ezrin.

ney epithelial cells. We propose these effects are due to the ability of ezrin to activate the small GTPase Rac1. We report herein that activation of the GTPase Rac1 pathway impairs the delivery of E-cadherin to adherens junctions during cell-cell contact assembly.

Production of ezrin T567D in nonconfluent MDCK cells increases the amount of GTP-bound Rac1, whereas the level of GTP-bound RhoA or Cdc 42 does not change. This suggests that ezrin functions upstream of Rac1 activation. How does ezrin activate Rac1? One possible mechanism is through an interaction between ezrin and the regulators of the GTPases. Indeed, a direct interaction between the FERM domain of ERM proteins and RhoGDI has been observed. It has been proposed that, by sequestering RhoGDI, ERM proteins allow the activation of the Rho GTPase by the exchange factors (Takahashi *et al.*, 1997). Hence, in MDCK cells producing ezrin T567D, activation of the GTPase Rac1 could be increased because the RhoGDI binding site on ezrin, normally cryptic in wild-type ezrin, would be unmasked in ezrin T567D. Another possibility by which ezrin may activate the small GTPase Rac1 is through the regulation of the exchange factors themselves. Indeed, an association of the FERM domain of radixin with the exchange factor Dbl has been observed in vitro (Takahashi *et al.*, 1998). In addition, ezrin might act as a scaffold by recruiting activated Rac1, or its exchange factor, to the membrane at the sites where actin remodeling takes place. ERM proteins have been found associated with the plasma membrane of MDCK cells, and this association was dependent on active Rho (Takaishi *et al.*, 1995; Kotani *et al.*, 1997). It is indeed important to stress that beside being present at the dorsal membrane of the epithelial cells, ezrin T567D is also present at the lateral membrane, whereas wild-type ezrin is localized mostly to the apical membrane. Alternatively, ezrin T567D might interact with a protein that is involved in the activation of Rac1. Interaction of hamartin with ERM proteins is required for the activation of the Rho pathway by serum or lysophosphatidic acid, which leads to cell adhesion to the substrate (Lamb *et al.*, 2000).

Concomitant to the activation of Rac1, we observed extensive formation of lamellipodia and loosening of the cell contacts. Our results show that the formation of lamellipodia in MDCK cells producing ezrin T567D is due to the activation of Rac1 because dominant negative Rac1N17 reinforced cell contacts and inhibited lamellipodia formation, whereas Rac1V12 induced membrane protusions and impaired E-cadherin localization at cell-cell contacts. This effect of Rac1 was prominent with cells grown at low density. Similar morphological changes were observed when using controlled expression of dominant active Rac1 in MDCK cells (Jou and Nelson, 1998) or in normal human keratinocytes (Braga *et al.*, 2000; Akhtar and Hotchin, 2001) grown at low density. Scattering of epithelial cells after treatment with stimuli such as growth factors, integrin engagement, or ligand binding to CD44 also involves Rac1 activation (Gimond *et al.*, 1999; Oliferenko *et al.*, 2000; Royal *et al.*, 2000). Interestingly, we have previously shown that ezrin is a downstream target of the HGF receptor and that it potentiates the effect of HGF on cell scattering (Crepaldi *et al.*, 1997). Thus, it is possible that the HGF-induced cell scattering is mediated by ezrin via the activation of Rac1.

However, in some experimental systems, activation of Rac1 was correlated with establishment of E-cadherin–dependent cell-cell adhesion (Braga *et al.*, 1997; Takaishi *et al.*, 1997; Ehrlich *et al.*, 2002). Expression of the Rac exchange factor Tiam 1 restored E-cadherin mediated cell-cell adhesion in MDCK cells treated with HGF or in Ras-transformed MDCK cells (Hordijk *et al.*, 1997; Sander *et al.*, 1998). Using a calcium switch method to follow junction assembly, it was observed that cadherin-dependent cell-cell contacts increased Rac1 activity (Nakagawa *et al.*, 2001; Noren *et al.*, 2001). One explanation to reconcile these results is that the effect of Rac1 on the junctional complexes might depend on the state of cell-cell contacts, i.e., their degree of maturity. This was recently demonstrated by following the distribution of a GFP-tagged active Rac1 mutant, at low cell density. Rac1 accumulated only at the newly formed cell-cell contacts, and not at older contacts (Ehrlich *et al.*, 2002). In line with this proposal, we have observed that when the cells expressing ezrin T567D were grown on filters, the level of GTP-bound Rac1 and the amount of cell surface E-cadherin were similar to those of control cells. Additionally, we observed that although cells expressing ezrin T567D failed to form tubules in collagen, cells grown on filters maintained a normal apical/basolateral polarity. These observations are corroborated by the observation that endogenous GTP-bound Rac1 is required for cyst formation in collagen, but not for maintenance of polarity on filters (O'Brien *et al.*, 2001).

The mechanism by which the small GTPase Rac1 interferes with assembly of E-cadherin is not known. Our results indicate that activated Rac1 inhibits the delivery of E-cadherin to the plasma membrane during cell-cell junction as-

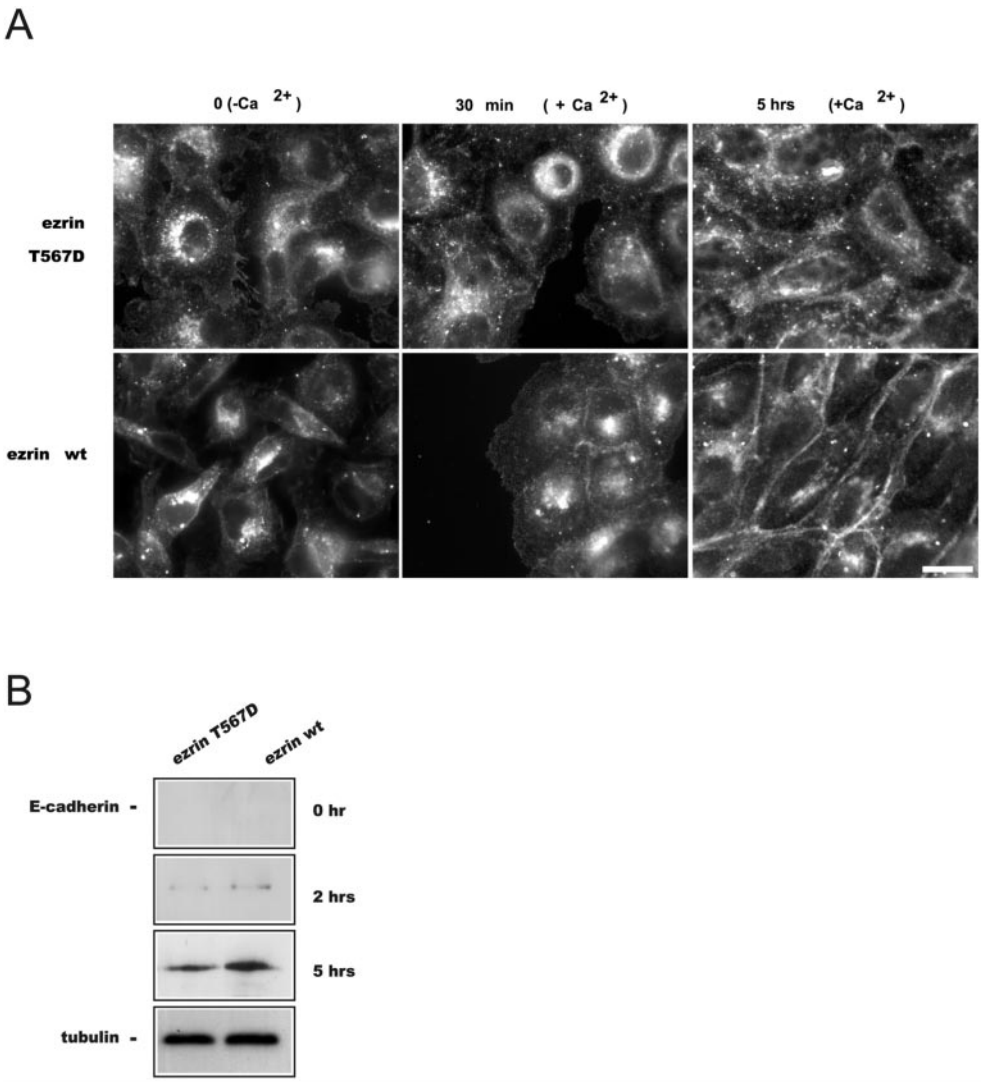

Figure 6. Ezrin T567D delays E-cadherin delivery to the plasma membrane. (A) MDCK cells cultured without doxycyclin for 4 d were plated on coverslips and grown in a low calcium medium for 4 h. Cells were then incubated in a calcium-containing medium for different periods of time. Cells were fixed and analyzed by immunofluorescence with an anti-E-cadherin antibody. Bar, 10 μm. (B) Biotinylated cell surface proteins from cells expressing wild-type ezrin or ezrin T567D were recovered on streptavidin beads and analyzed by immunoblotting with an anti-E-cadherin antibody. Immunoblotting with an anti-tubulin antibody served as loading control.

sembly of MDCK cells producing ezrin T567D. Because neither the amount nor the half-life of E-cadherin changed in MDCK cells producing ezrin T567D compared with control cells, the decrease in the amount of E-cadherin at the plasma membrane results from the accumulation of this protein in intracellular compartments. These observations are in agreement with previous reports implicating activated Rac1 in the regulation of membrane protein traffic in MDCK cells and keratinocytes (Lamaze *et al.*, 1996; Jou *et al.*, 2000; Akhtar and Hotchin, 2001).

Our results indicate that activated Rac1 regulates E-cadherin trafficking in subconfluent cells expressing ezrin T567D because in confluent cells the level of active Rac1 was similar to that of control cells. Yet, we have observed a decrease in the rate of E-cadherin endocytosis in confluent cells. This suggests that ezrin T567D might control some steps of protein transport in addition to its role in Rac1 activation. In line with this hypothesis, it has been proposed that the interaction of ezrin with EBP50 is involved in the recycling of the β2-adrenergic receptor (Cao *et al.*, 1999).

In conclusion, our observations indicate that ezrin plays a role in the transition from polarized epithelial cells to more "spread out" cells by regulating the transport of E-cadherin to the plasma membrane. This function implicates the activation of the small GTPase Rac1 by ezrin and might involve a direct effect of ezrin on the membrane transport machinery. Elucidating the mechanisms by which ezrin controls E-cadherin delivery to the plasma membrane may be relevant to understand its emerging role in tumor progression (Gautreau *et al.*, 2002).

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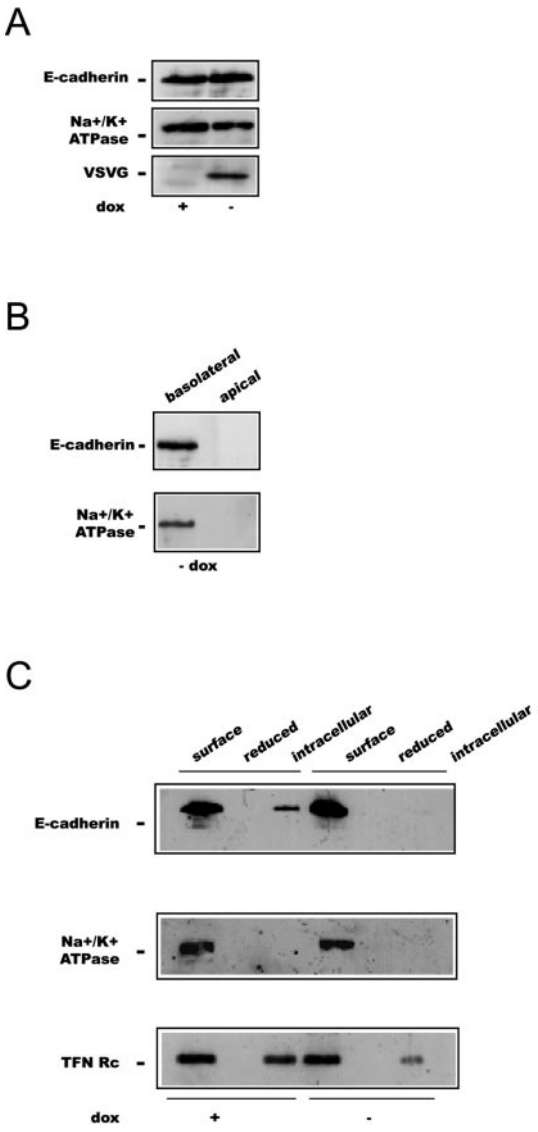

Figure 7. Ezrin T567D slows down E-cadherin internalization in polarized monolayer. (A) Confluent MDCK cells grown on filters in the presence (+ dox) or absence (− dox) of doxycyclin were surface-biotinylated at 0°C from the basal pole. Surface-biotinylated proteins were recovered from cell extracts on streptavidin beads and analyzed by immunoblotting with anti-E-cadherin or -Na^+/K^+ ATPase antibodies. Ezrin T567D expression was detected in the absence of doxycyclin by immunoblotting with a VSVG antibody. (B) Confluent MDCK cells grown on filters in the presence or absence of doxycyclin were surface biotinylated at 0°C either from the apical or basal pole. Surface-biotinylated proteins were recovered from cell extracts on streptavidin beads and analyzed by immunoblotting with anti-E-cadherin or -Na^+/K^+ ATPase antibodies. (C) Internalization of surface-biotinylated E-cadherin. Confluent MDCK cells grown on filters in the presence or absence of doxycyclin were surface biotinylated at 0°C (surface) and either stripped with glutathione immediately after biotinylation (reduced) or allowed to be internalized at 37°C for 1 h and stripped (intracellular). Biotinylated proteins were recovered from cell extracts on streptavidin beads and analyzed by immunoblotting with anti-E-cadherin, anti-Na^+/K^+ ATPase, or anti-transferrin receptor.

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